RED BLOOD CELL DENSITY AND VOLUME CHANGES IN MEN EXPOSED TO HYPOBARIC HYPEROXIA

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13. ABSTRACT

Eight volunteers were exposed to 100% O_2 at 258 mm. Hg for 31 days. Blood samples were taken daily, and red cell (RBC) volume distributions were determined by Coulter counter--RBC density distributions, by the method of Danon and Marikovsky. RBC volume distribution varied in a cyclic fashion but exhibited no consistent, significant changes. After 4 to 6 days in the test environment, the RBC populations showed an increase in the proportion of dense cells; and a trend of increasing senescence was evident for the remainder of the exposure period. Postexposure changes indicated the formation of new red cells.

I

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II

RED BLOOD CELL DENSITY AND VOLUME CHANGES IN MEN EXPOSED TO HYPOBARIC HYPEROXIA

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III

FOREWORD

This report was prepared in the Cellular Physiology Branch of the Environmental Sciences Division under task No. 793002 and NASA Defense MIPRT-74401-G. The work was accomplished between 1 July 1970 and 15 December 1971. The paper was submitted for publication on 13 November 1972.

The voluntary informed consent of the subjects used in this research was obtained in accordance with AFR 80-33.

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This report has been reviewed and is approved.

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Commander

TV

RED BLOOD CELL DENSITY AND VOLUME CHANGES IN MEN EXPOSED TO HYPOBARIC HYPEROXIA

I. INTRODUCTION

Among the reported in vivo effects of hyperoxia on erythrocytes are alterations in the cell's biophysical properties resembling those changes associated with normal red blood cell (RBC) aging. These alterations include increases in osmotic fragility, in cell density, and in rate of agglutination by polycations (3). Other physical changes attributed to normal senescence of erythrocytes (such as decreases in reversible deformability and in cell volume) have received little or no experimental attention in other investigations of hematologic responses to elevated oxygen tension. A comprehensive 80-day study--during which 8 healthy young men were exposed to 100% O_2 at 258 mm. Hg total pressure for 31 days--recently determined RBC populations in relation to osmotic fragility, density, and cell volume. Hence a report has already presented results of the osmotic fragility measurements as related to changes in active potassium flux and in organic phosphate levels (4).

II. MATERIALS AND METHODS

Overall details of the research equipment, procedures, and subject selection are available in a previous report (4). In brief, however, 8 USAF airmen (informed volunteers), 18 - 20 years old and weighing 63 - 82 kg., were confined in a test chamber for, sequentially: a 31-day control period, at normal atmospheric conditions; a 31-day exposure, to 100% O_2 at 258 mm. Hg total pressure (actual composition in mm. Hg partial pressure: O_2, 253 $\pm$ 3; N_2, 0.5 $\pm$ 0.5; CO_2, 1.5 $\pm$ 0.5); and a 13- to 18-day controlled recovery period, in a normal atmosphere. Diet was controlled throughout the experiment, with caloric intake being adjusted to each subject's activity level. In the pre-exposure control period, all subjects participated in a uniform work-load exercise program (measured by bicycle ergometer). Of the 8 subjects, 4 continued at this same activity level for the duration of the study; but the other 4 were assigned to modified bed rest from the beginning of hypobaric hyperoxic exposure through the recovery periods. Daily blood samples, averaging slightly less than 11 ml., were required from each subject. These samples were taken routinely between 0700 and 0900 hours, local time. The average interval between the measurements reported herein was 4 days. Technical problems delayed the initiation of RBC volume distribution determinations until the second week of the control period.

For volume distributions, a Coulter counter (model B) with automatic particle-size distribution plotter was used. The instrument knob

settings, utilizing a 100 μm. orifice, could be adjusted for: "1/aperture current = 1/2; 1/amplification = 1/4; and matching switch = 64-H." Phosphate-buffered saline (pH 7.4, 300 milliosmoles/liter, membrane-filtered), prepared according to the directions of Coopersmith and Ingram (1), was used to suspend heparinized whole blood at a 1:50,000 dilution for both RBC counting and sizing. From the volume distribution plot and the RBC count were calculated both the proportionate numbers and the proportionate percentages of the total population within defined volume ranges.

The procedure of Danon and Marikovsky (2) was used to obtain density distributions of RBC populations. For each density distribution determination, well-mixed heparinized blood was introduced over each of 20 immiscible separating fluids (of differing but known specific gravity) in microhematocrit tubes. The tubes containing blood and separating fluid were then placed in a hematocrit centrifuge and processed at 11,000 r.p.m. for 30 min. A linear scale with closely spaced subdivisions was used with an illuminated magnifying lens to measure the length of the RBC columns above and below the clear layer of separating fluid. From these readings was calculated the volume percentage of the total RBC population passing through each separating fluid. A plot of these percentages vs. density (S.G.) provided a density distribution curve with 20 points. The battery of 20 separating fluids, ranging from 1.062 - 1.138 S.G. in increments of 0.004 S.G., was prepared by mixing dimethyl phthalate and dibutyl phthalate in the proportions given by Danon and Marikovsky (2). These investigators reported displacement of the density distribution curve toward higher density at an approximate rate of 0.008 S.G. units in 5 hours for blood samples kept at room temperature. This artifact was eliminated by keeping the blood at 4° C. So, in the present study, small siliconized tubes containing heparinized blood were kept in ice from the time of sample collection until processing began.

To facilitate data processing and presentation, both the density distribution and the volume distribution curves were divided into five component fractions. For the volume distribution, four of the fractions were of equal volume increments (28 μ^3); but a fifth fraction (largest cells) spanned a 57 μ^3 range (140.5 - 197.6 μ^3), and thus contained only about 2.5% of the total population. The three central fractions of the density distributions were of equal density increments (0.008 S.G.). The least dense fraction was defined at its upper limit (i.e., less than 1.094 S.G.)—the most dense fraction, at its lower limit (i.e., greater than 1.118 S.G.). The combined contribution of these two extreme fractions averaged less than 10% of the total RBC population. In the statistical analyses, each density and volume fraction was treated as a separate variable. With values calculated as percentages of the total population, however, these variables were not independent; for an increase in one fraction must be compensated by the decrease in one or more of the other fractions, so that the sum of all five fractions will always equal 100%.

Statistical tests on the data included a repeated measurements analysis of variance for each variable within each experimental period (control, exposure, and recovery) and a set of five paired Student's t-tests on certain early and late period means. In addition, the selected early and late period means were subjected to the Wilcoxon signed rank test, a nonparametric test analogous to the paired t-test, to determine the degree of conformity of each subject to the change indicated by the group mean difference. Statistically significant probability levels in both the t-test and the signed rank test indicated not only a significant mean difference but also individual changes in the same direction by the majority of subjects.

III. RESULTS AND DISCUSSION

In general, the statistical analyses showed no significant differences between active and inactive subjects insofar as density and volume distributions of RBC populations were concerned. Hence the results presented in figure 1 and table I are based on means of all 8 subjects. Designation of population fractions is in accordance with the widely held view of the density:volume:age relationship---that is, young RBCs are less dense and larger than old RBCs. Thus, with increasing fraction number, density increases and volume decreases. The descriptive terms "youngest" and "oldest" were placed within quotation marks (fig. 1; table I) in recognition of electron microscopic evidence (3) indicating that structural alterations in membranes of red blood cells subjected to hyperoxia in vivo were not identical with those of normal physiologic aging. The age terms were further qualified (as indicated by question marks in fig. 1 and table I) in the volume distribution context; for the present study provided little evidence (real or apparent) of an RBC age: size relationship.

Grossly, the paired plots of percentages of the mean RBC population within each density and volume fraction vs. time in days (fig. 1) reflect the differences in shape of the distribution curves. The characteristic non-Gaussian distribution pattern of normal human RBC volumes, as obtained by the Coulter method, is evident in the appearance of approximately two-thirds of the population at the small end of the volume range (fractions IV and V). Although somewhat skewed, the mean density distribution curves approach a normal distribution pattern with roughly 50% of the population in the central fraction III.

Visual comparison of the paired plots (fig. 1) reveals greater stability with treatment and time in the mean volume distribution as contrasted with the obviously changing pattern of density distribution. The latter shows a sharp rise in the proportion of less dense cells (fractions I and II) early in the control period, with the subsequent establishment of a new and relatively stable mean control population that may have been younger than normal. After a few days under hypobaric hyperoxic conditions, percentages of less dense cells decreased

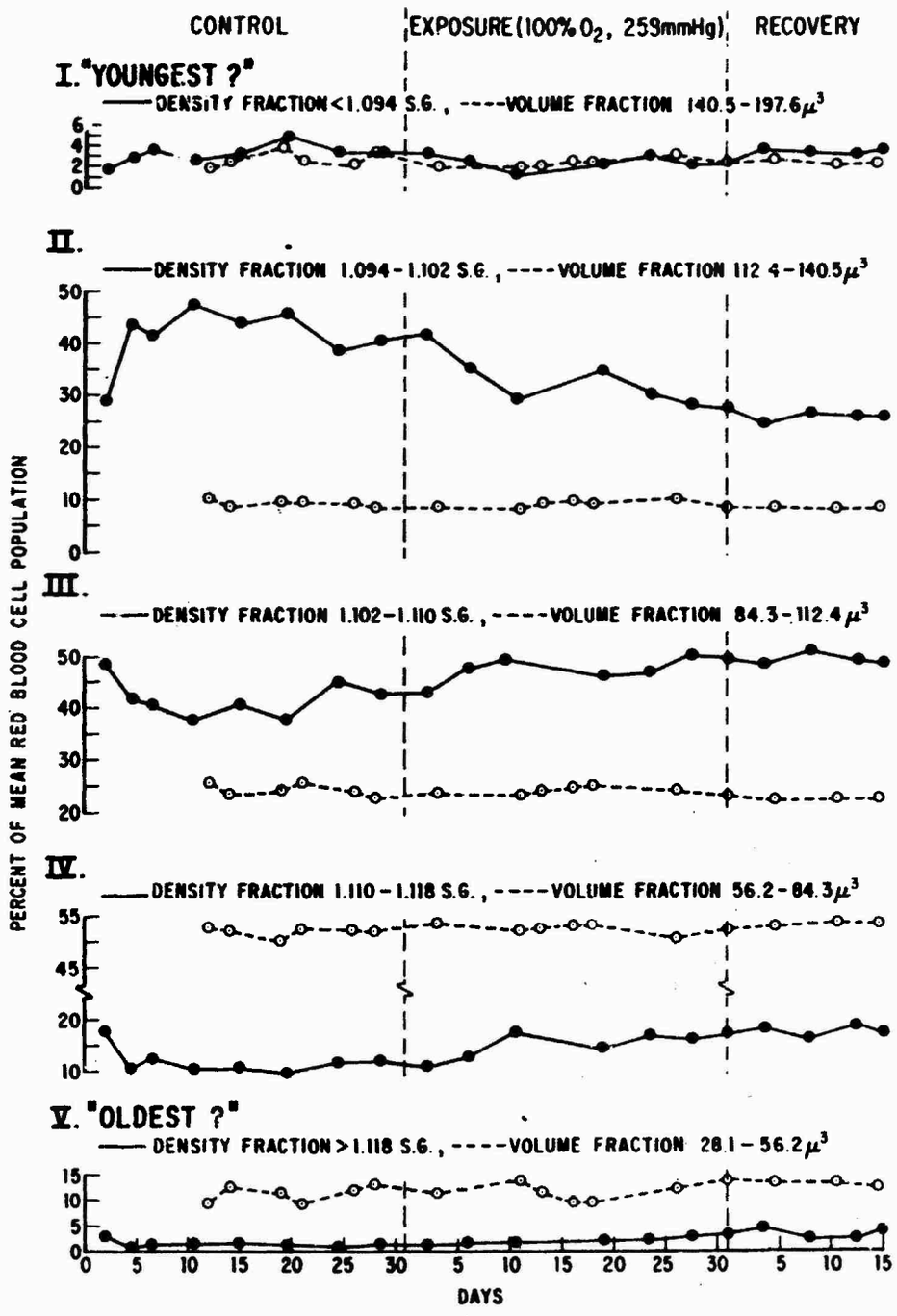

FIGURE 1

Variation of the proportion of total RBCs in five ranges of density (solid circles) and volume (hollow circles) during control, O_2 exposure, and recovery periods.

(proportions of more dense cells increased) and continued to do so with some variation throughout the exposure period. The mean density distribution at end of exposure was similar to that in the initial control period. The trend towards increasing mean cell density continued into the early recovery phase before leveling off.

Changes within the control period were statistically significant for three density fractions (II, IV, and V) as tested by the analysis of variance (table I). With the possible exception of the most dense fraction (V), however, none of the density fractions showed statistically significant differences in the paired t-test comparisons of early and late control period means. The late control period percentage for fraction V was smaller than the corresponding early control value, but at a probability level of only 0.1; and the significance of this difference was further diminished by the absence of a consistent trend among the individual subjects as measured by the signed rank test. For the exposure period, the analysis of variance tests indicated significant alterations in density fractions II, IV, and V. The inference of altered density distributions, resulting from sustained hypobaric hyperoxic exposure, gains additional statistical support in the comparisons of late exposure means with late control and early exposure means for fractions II, IV, and V. The case for meaningful changes in the minor (< 5% of population) and most dense fraction (V) is weakened somewhat by an interaction component (Activity X Time), significant at the 0.05 level in both the analysis of variance and the late control vs. late exposure comparison. But the elevated level of this fraction (V) at the end of the exposure period, as compared with the early exposure mean, is highly significant ($P < .001$ paired t-test; $P < .01$ signed rank test). It was in this comparison (early exposure vs. late exposure) that the central large fraction III showed a difference with statistical significance ($P < .05$). The within-period changes during recovery were significant only in the most dense fraction V ($P < .01$ analysis of variance).

In contrasting late control means with late recovery means, however, a lower percentage of fraction II ($P < .05$) and higher proportions of the dense fractions IV and V ($P < .05$) were indicated at the end of the recovery period (table I). The one significant density change within the first few days of recovery occurred in the least dense fraction I, which showed an increase--from a mean value of 2.4% on the last day of exposure--to 3.7% on about the third day of recovery ($P < .05$). This change coincides with the finding of marked reticulocytosis immediately postexposure (4), thus further confirming the well-established RBC density:age relationship.

In contrast to the marked changes in density distribution patterns, the volume distribution measurements indicated few significant alterations in the proportions of the five volume fractions (table I). These important alterations were found in only two fractions (III and V) and, with one exception, occurred only in the control period. The exception ($P < .05$), possibly attributable to hyperoxia, was found in the exposure

TABLE I

Statistical tests of significance for density and volume distribution changes of mean RBC populations (from 8 men, exposed 31 days to 100% O_2 at 258 mm. Hg)

Population distribution (Fraction No. & range)	Within-period analysis of variance (Probability level)			Comparison of early and late period means (Probability level: Paired t-test / Probability level: Signed rank test)				
	Control	Exposure	Recovery	EC vs. LC	LC vs. LE	EE vs. LE	LE vs. ER	LC vs. LR
Density distribution:								
I "Youngest" 1.094 S.G.	-	-	-	- / -	- / -	- / -	< .05 / < .05	- / -
II 1.094 - 1.102 S.G.	< .01	< .001	-	- / -	< .10 / < .05	< .05 / < .05	- / -	< .05 / < .01
III 1.102 - 1.110 S.G.	-	-	-	- / -	- / -	< .05 / < .05	- / -	- / -
IV 1.110 - 1.118 S.G.	< .01	< .01	-	- / -	< .05 / < .05	< .05 / < .01	- / -	< .05 / -
V "Oldest" > 1.118 S.G.	< .01	< .001*	< .01	< .10 / -	< .01* / < .01*	< .001 / < .01	- / -	< .05 / < .05

Volume distribution:									
I "Youngest?" 140.5-197.6 μ^3	-	-	-	- / -	- / -	- / -	- / -	- / -
II 112.4 - 140.5 μ^3	-	-	-	- / -	- / -	- / -	- / -	- / -
III 84.3 - 112.4 μ^3	< .001	-	-	< .05 / < .05	- / -	- / -	- / -	- / -
IV 56.2 - 84.3 μ^3	-	-	-	-† / -†	- / -	- / -	- / -	- / -
V "Oldest?" 28.1-56.2 μ^3	< .01	< .05	-	< .10 / -	- / -	- / -	- / -	- / -

EC = early control, and LC = late control; EE = early exposure, and LE = late exposure; ER = early recovery, and LR = late recovery.

S.G. = percentages vs. density

*Activity X time interaction significant at 0.05 level.

†Active group vs. inactive group difference significant at 0.05 level.

period analysis of variance for the fraction of smallest cells, fraction V. The fact that a sustained period of relatively low percentages of this fraction occurred between days 10 and 30 of the exposure period (fig. 1) may indicate preferential attrition of small RBCs during prolonged hyperoxia. The control period changes shown to be significant in the analysis of variance for fractions III ($P < .001$) and V ($P < .01$) resembled inversely related oscillations (fig. 1), but control data were insufficient to establish clearly a normal periodicity in volume distribution of RBC populations. Statistical comparison of early and late control period means showed a significant difference at the 0.05 level for volume fraction III in both the paired t-test and the signed rank test. This comparison of early control vs. late control period means for fraction IV provided the only instance of a statistically significant difference ($P < .05$) between the groups of active and inactive subjects.

The means of total RBC counts, as obtained concurrently with the volume distributions, varied markedly with time in all periods. This phenomenon is shown in figure 2, along with single plot condensations of the percentage distributions of the mean RBC population, volume, and density. Because of these frequent acute changes in mean total count, most of the within-period analysis of variance tests using mean cell numbers in each of the five volume fractions were statistically significant, whereas the proportions of these fractions were relatively stable (fig. 2). Statistical comparisons of early and late period mean cell numbers, however, were significant only in the tests of early control vs. late control for fractions II, III, and IV. The pronounced variations with time in measured RBC counts remain unexplained. Limitations in current methods permitted only three measurements of total plasma volume (5): the first, at the end of the control period (mean of all subjects, 3429 ml.); the second, in the fourth week of exposure (mean, 3269 ml.); and the last, in the second week of recovery (mean, 3265 ml.). This change of less than 5% in mean plasma volume over a period of weeks is in marked contrast to 10% - 20% variations in mean total RBC count occurring at 2-day intervals.

Coopersmith and Ingram (1), in a careful study of the age:density:volume relationship of dog erythrocytes, labeled a cohort of RBCs with ^{59}Fe. They found newly produced cells at the top of a column of centrifuged red cells, and a progressive downward migration in the column as the labeled cells aged in vivo. Their study confirmed several earlier investigations, showing increasing cell density with increasing age of both human and rabbit erythrocytes. However, determinations of volume distributions in four fractional portions of the RBC populations having different mean densities (different mean ages), in the Coopersmith and Ingram study, showed no significant volume alteration in the normal dog erythrocyte throughout the entire life-span of the cell. These investigators have cited a number of conflicting reports on the age:cell volume relationship of human erythrocytes. The present study provided results essentially in agreement with the findings of Coopersmith and Ingram, although the subject species and experimental conditions were different.

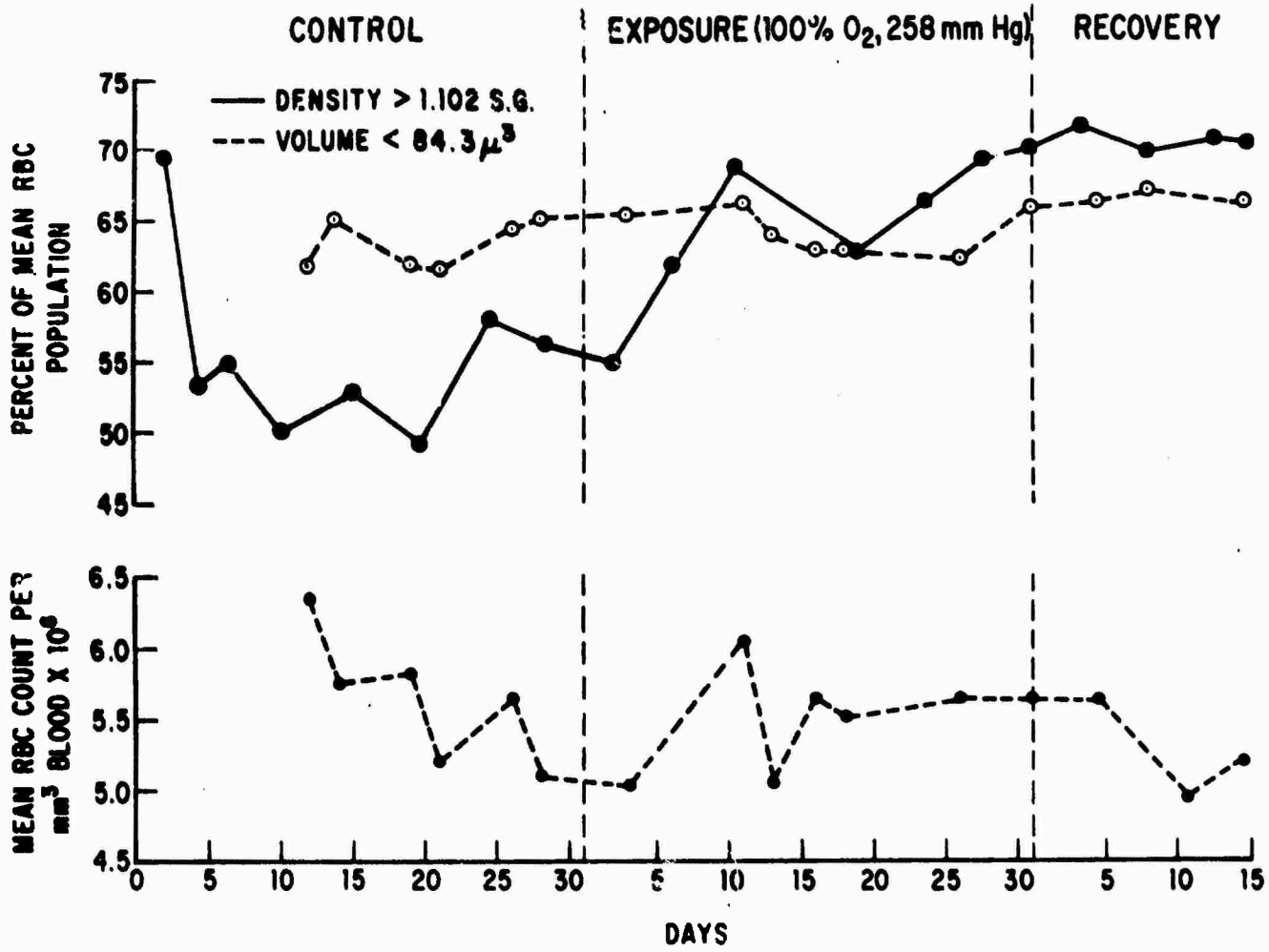

FIGURE 2

Volume (hollow circles) and density (solid circles) distribution of total RBCs, expressed as absolute counts and as percentages of the total number for control, O_2 exposure, and recovery periods.

Electron microscopic examinations by Danon et al. (3) showed structural differences in the membranes of normal physiologically old cells as compared with damaged membranes of RBCs from rabbits exposed in vivo to elevated oxygen tensions. Thus, assuming a volume:age relationship in human erythrocytes, our finding of no significant alteration in volume distributions of RBCs in humans subjected to moderate hyperoxia may be interpreted as a second point of difference between normal aging and oxygen-induced "premature aging."

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